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Use of calcein to estimate and validate age in juveniles of the winged pearl oyster *Pteria sterna*

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Abstract – Determining age is an important step when assessing growth, mortality, and yield of cultivated and wild populations, but studies linking shell growth marks in the pearl oyster *Pteria sterna* with the age of individuals are lacking. Thirty juveniles (20.0 ± 1.2 mm shell height), collected from a winter spatfall, were marked with the fluorochrome calcein and kept in the field in culture containers. After day 16, the juveniles were cleaned and their shells cut along the sagittal axis to determine periodicity of micro growth bands formed in the inner shell layers and to estimate age. During this trial, fluorescent calcein marking succeeded in individuals larger than 20 mm shell height; these formed an average of 15 micro growth bands over the 16 days, representing 1 band per day. The marker created a wide fluorescent band containing three micro growth marks, suggesting that calcein was incorporated into the shell over the first three days. The use of calcein was found to be an accurate method for validating the micro growth band frequency of formation in *P. sterna* juveniles, which in turn can help to estimate age.

Key words: Pearl oysters / Age estimation / Age validation / Fluorochrome calcein marker

1 Introduction

In marine bivalves, estimating the age of individuals is a key task for assessing their growth, biomass yield, and mortality in cultivated and wild populations. In the Pteriidae family (pearl oysters), growth and age have been studied for the main pearl producing species, including *Pinctada fucata* (Pandya 1976; Chellam 1978; Velayudhan et al. 1996), *P. margaritifera* (Southgate and Beer 2000; Pouvreau et al. 2000), *P. maxima* (Gervis and Sims 1992; Lee et al. 2008), *P. mazatlanica* (Gaytán-Mondragón et al. 1993; Monteforte and Garcia-Gasca 1994; Saucedo and Monteforte 1997a; Saucedo et al. 1998), and *Pteria sterna* (Araya-Núñez et al. 1991; Bückle-Ramírez et al. 1992; Del Rio-Portilla et al. 1992; Monteforte et al. 1995). In most of these studies, age estimations were made by assigning an average date at the moment when spat were separated from artificial collectors and tagged prior to starting the nursery period. This process, however, produces errors because settlement is a time-dependant process and individuals are recruited on artificial collectors at different times, resulting in a mixture of ages within the same capture cohort. In a few cases, age has been determined by counting the number of growth bands or rings appearing on the shell surface at certain times. This was done for cultivated *P. fucata* and *P. maxima* (Pandya 1976; Gervis and Sims 1992) as well as in wild mussels and clams (Seed and Brown 1978; Shafee 1992). Interestingly, the frequency of ring formation in the shell was not validated in these studies. The presence of growth rings has not been reported by other studies on pearl oyster species in different locations, such as *P. margaritifera* from the Cook Islands (Sims 1993) and *P. maxima* from Australia (Hynd 1960).

In pearl oysters, the shell is subjected to strong erosion caused by abiotic and biotic factors. Among the biotic factors, the presence of drilling organisms, such as sponges, tunicates, corals, anemones, polychaete worms, and gastropods are particularly relevant, as they make the identification of growth rings difficult (Monteforte et al. 1995; Taylor et al. 1997; Guenther and De Nys 2006). An alternative means of measurement, which overcomes this problem, is to search for age marks in the inner layers of the shell. One option is to assess age through growth rings in the inner layers of the thickening shell, which are known as "micro growth bands" or "marks" (Anwar et al. 1990). However, studies of micro growth bands are scarce for pearl oysters and inexistent for *P. sterna*.

One of the major problems of determining age using micro growth bands is the absence of appropriate validation studies. Validating absolute age is equivalent to accurately estimating age (Campana 2001). Marking techniques are presently

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available that use substances to create a fluorescent mark in the shell. This approach not only proves to be useful for validating the periodicity of micro growth bands, but provides new criteria for estimating age in marine bivalves (Kaehler and McQuaid 1999; Moran 2000; Riascos et al. 2007; Linard et al. 2011).

This study investigated the presence of micro growth bands in the inner shell structure of juvenile *P. sterna* (Gould 1851) collected from natural spatfall. When the bands occurred, their periodicity was tracked using the fluorochrome calcein as a marker.

2 Materials and methods

2.1 Origin and handling of specimens

On 13 December 2008, several spat collectors, made from 30 cm^2 of dark plastic mesh with 0.5 cm^2 mesh size, were attached to a long-line system used for cultivating pearl oysters in Bahía de La Paz, Baja California Sur, Mexico (24° 16′ N, 110° 19′ W). December is usually the beginning of the natural reproductive season for *P. sterna* in this area (Saucedo and Monteforte 1997b). Collectors were removed on 28 March 2009 after 108 days in the water. After separating the spat, 30 young individuals (20.0±1.2 SE mm shell height), covering the size range of oysters recruited from collectors, were selected for the marking experiment. These oysters were measured for dorsoventral distance (shell height), anteroposterior distance (shell length) and shell width using a SCALA digital calliper (0.1 mm), and weighed to obtain total wet weight using an analytic OHAUS scale (0.001 g).

2.2 Marking with fluorochrome calcein

The collected juveniles were injected in the paleal cavity with a solution of seawater containing 125 mg L^{-1} fluorochrome calcein (hereafter referred to as calcein; Sigma, Chem. Abs. # 1461-15-0), following the methodology of Kaehler and McQuaid (1999). Solution was injected into the paleal cavity until overflow occurred (~0.5 ml). Individuals were tagged on the shell with plastic labels and then placed in commercial cultivation containers kept at 15 m depth on an underwater trestle of a pearl farm (Perlas del Cortez) in Bahía de La Paz, where they remained for 16 days. No acclimation was needed because the artificial collectors and underwater trestle were in the same study area and, therefore, in the same environmental conditions. Water temperature varied between 20.5 and 22.9 °C and there were two tides each day.

When the field period ended, individuals were collected and re-measured for shell height, length, width and total wet weight. The shells were separated, cleaned of fouling matter, dried and cut with a low-velocity cutter (Buehler Isomet with an Ukam disc and $3 \times 0.006 \times 1/2''$ diamond blade). The cut was made along the sagittal axis, from the umbo to the upper margin, and produced thin, 320 μm shell slices, which were polished with 30, 12, and 3 μm lapping film (3M, St. Paul, MN) on both sides. Each shell piece was mounted on a slide with cyanoacrylate glue and observed under an Olympus BX-41 fluorescence microscope. Shell slides were digitized with a CoolSnap digital camera (Media Cybernetics, Bethesda, MD), and analyzed with Image Pro Plus 5.1 software (Media Cybernetics). To observe the calcein mark, each slide was excited with 450 to 480 nm light (Leica, Filter U-MWB2) and observed at 20×. To determine the number of micro growth bands, the slides were observed under a phase-contrast microscope at 20×, and 100× to study the ultrastructure of the micro growth bands. We also looked for autofluorescence within the range 330 to 385 nm, using a Leica U-MWU2 filter.

3 Results

3.1 Increase in absolute size and weight

From an initial size of 20.5 ± 1.2 mm (height), 17.5 ± 1.1 mm (length), 7.8 ± 0.2 mm (width), and 1.90 ± 0.06 g (total wet weight), individuals increased in height (4.0 ± 0.2 mm), length (2.5 ± 0.2 mm), width (1.6 ± 0.04 mm), and total wet weight (0.8 ± 0.01 g) over the 16 days (Fig. 1). This increase represents an average growth rate of 0.27 mm d^{-1} (shell height), 0.17 mm d^{-1} (shell length), 0.11 mm d^{-1} (shell width), and 0.055 g d^{-1} (wet weight).

3.2 Marking with fluorochrome calcein

The calcein marking procedure did not affect short-term survival of juvenile oysters, since 29 out of 30 survived to the end of the experiment. Additionally, there were no visible alterations in the shell to suggest a reaction to the dye. However, 11 individuals (38%) did not display any fluorescent marking and the remaining 18 (62%) were mostly individuals with shells >20 mm height at the time of the marking.

Individuals that incorporated the calcein developed a mark that was clearly visible under the fluorescence microscope in two regions, the middle section of the shell slice (Fig. 2a), and close to the end of the curve of the umbo (Fig. 2b). We also observed auto fluorescence in the shells, which appeared either as a series of thin, separated bands (Fig. 3a) or as a thick, continuous band (Fig. 3b).

3.3 Inner micro growth bands

A very distinctive arrangement of alternating clear and dark bands was observed in the shells, with the clear bands being wider than the dark bands (Fig. 4 and 5). The estimation of the number of micro growth bands involved pairs of clear and dark bands, we only counted the dark bands. The bands were easily distinguishable in the umbo region when using the ph2 filter under the phase-contrast microscope (Figs. 4 and 5).

The calcein mark found inside the shell was wide and included several micro growth bands. There were 3.47 ± 0.04 growth bands counted within the fluorescence mark and 11.79 ± 0.24 bands outside the mark, yielding a total of 15.29 ± 0.26 micro growth bands during the 16-day trial (Figs. 4a, 4b).

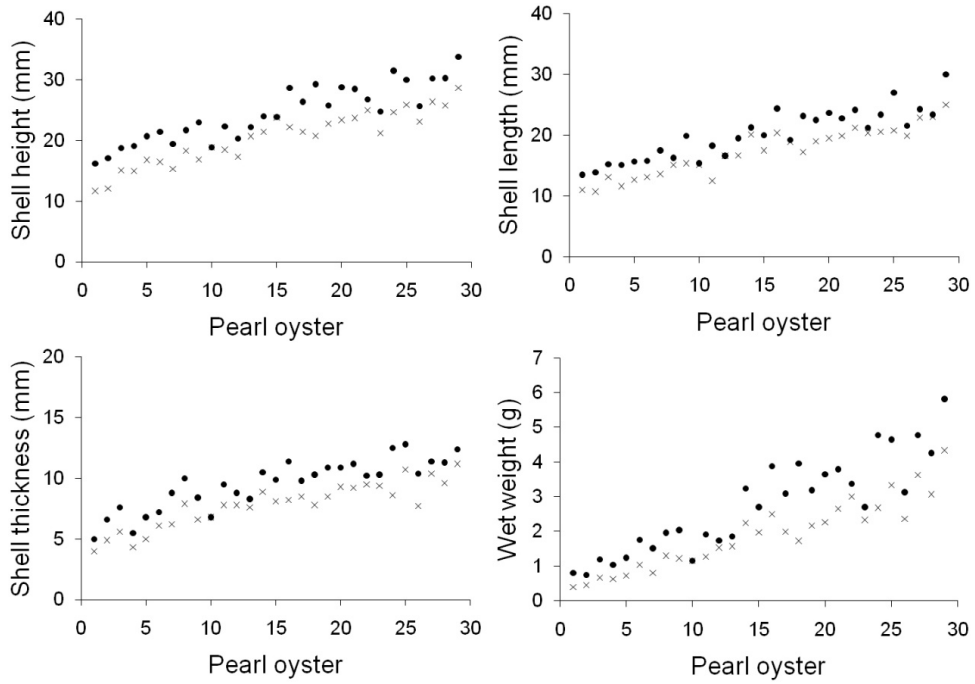
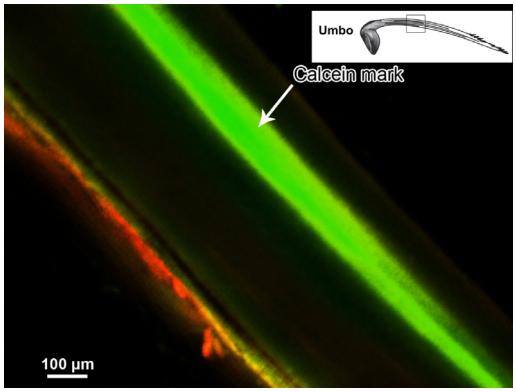
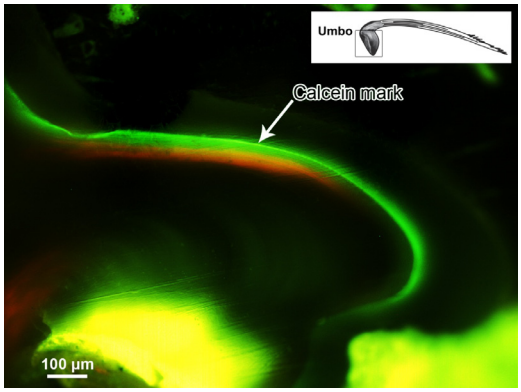


Fig. 1. Initial (× dot) and final (black dot) values of shell size and total wet weight of *P. sterna* juveniles during the trial.

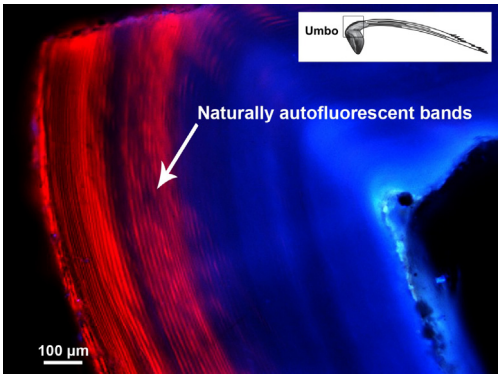


(a)

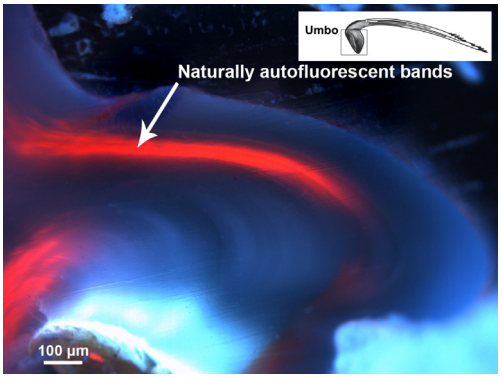


(b)

Fig. 2. Calcein mark present in the middle section (a) and in the umbo region (b) of the shell of *P. sterna* juveniles. Images taken under a fluorescence microscope at 450 – 480 nm (10×). The inset shows the approximate position of the image on the shell slice.



(a)



(b)

Fig. 3. Two examples of autofluorescence in the umbo region of the shell of *P. sterna* juveniles showing as thin separated bands (a), and as a thick continuous band (b) under the fluorescence microscope at 330 to 385 nm (10×). The inset shows the approximate position of the image on the shell slice.

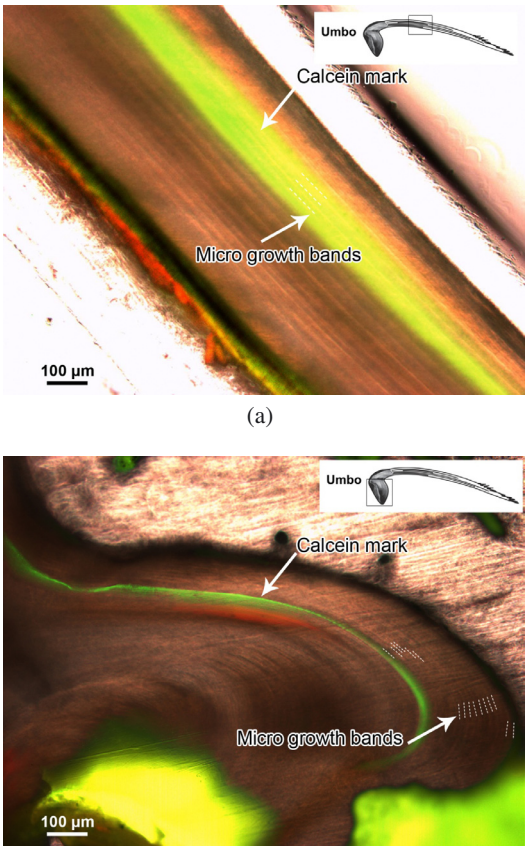


Fig. 4. Composite of two images of a single shell slice (10×) in the middle section (a) and in the umbo region (b), showing the calcein mark and the 16 micro growth bands formed between the beginning and end of the experiment (emphasized with added lines). The inset shows the approximate position of the image on the shell slice.

This pattern suggests that the formation of micro growth bands occurs daily. If this is the case, then the possible range of micro growth bands in the marked individuals is between 17 (minimum value) and 124 (maximum value), as counted from the day the artificial collectors were immersed in the water up to the end of the marking experiment. In our sample, we observed a minimum of 29 bands (16.2 mm shell height) and a maximum of 110 micro growth bands (33.8 mm shell height), which is consistent with the above range of values. Additionally, there was a significant positive relationship between the total number of micro growth marks and the size of the individuals ($r = 0.93$, $p < 0.05$) (Fig. 6).

The ultrastructural analysis of a micro growth band revealed the presence of several sub-daily bands, which were observed as a narrow package of lamellae (Fig. 7). A total of 12 lamellae were counted per layer (Fig. 7). This pattern was clearly visible in two shell slices that also had the best observable micro growth bands of those studied.

4 Discussion

Injection of calcein is toxic and can cause death in some groups of marine invertebrates, including brachiopods

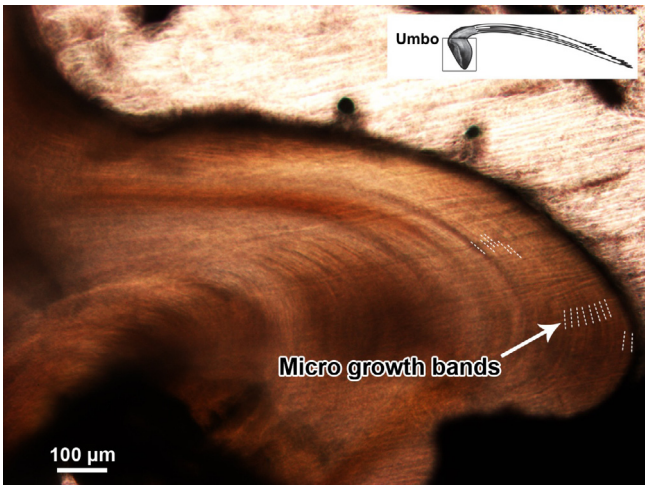


Fig. 5. Micro growth bands in the umbo region observed under a phase contrast microscope (10×). The inset shows the approximate position of the image on the shell slice.

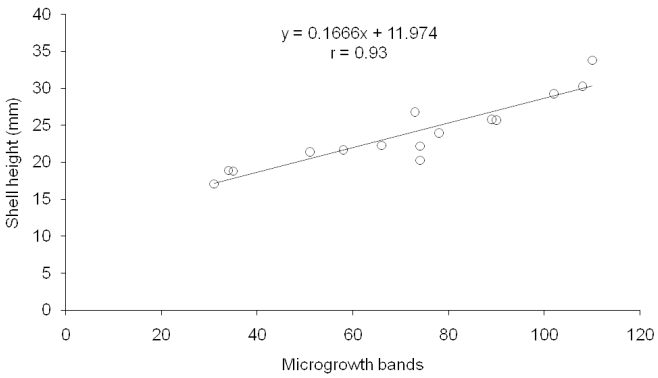


Fig. 6. Lineal regression between the total number of micro growth bands in the shell and the increase in size of individual juveniles by the end of the trial.

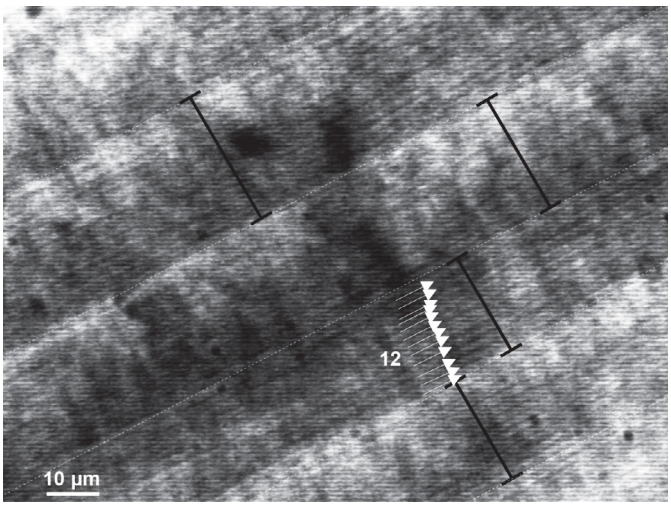


Fig. 7. Daily micro growth bands (marked in black) and tabular aragonite structures (marked with arrows) in the nacreous layer of the shell of *P. sterna* juveniles observed at 100×.

(Rowley and Mackinnon 1995) and abalone (Day et al. 1995). In marine molluscs, the toxic effect of calcein has not been reported for other tested species such as *Perna perna* (Kaehler and McQuaid 1999), *Nucella ostrina* (Moran 2000), *Concholepas concholepas, Mesodesma donacium* (Riascos et al. 2007), and *Donax hanleyanus* (Herrmann et al. 2009). In this study, only one of 30 oysters died (likely from predation since the shell was not found), suggesting that a calcein concentration of 125 mg L^{-1} is not toxic to juvenile *P. sterna* and does not affect their short term survival.

The incorporation of a fluorescent mark in the shell of young *P. sterna* indicates that calcein can be a useful tool for determining and validating the periodicity of growth bands. Other studies of marine bivalve species have also reported success in the use of calcein as a marker: *P. perna* (Kaehler and McQuaid 1999), *Adamussium colbecki* (Heilmayer et al. 2005; Lartaud 2010), *M. donacium* (Riascos et al. 2007), *D. hanleyanus* (Herrmann et al. 2009), *Cerastoderma edule* (Mahé et al. 2010) and *P. margaritifera* (Linard et al. 2011). However, in most of these studies, injection of calcein has been used for estimating absolute growth rather than for estimating age or counting micro growth bands.

In the present experiment, the fluorescent marker was only visible in 62% of the tested specimens, all of which were larger than 20 mm at marking time. This success rate is similar to that reported by Linard et al. (2011) for *P. margaritifera*, where only 65% of the shells injected with calcein revealed a clear mark. This result could reflect a differential rhythm of calcium deposition during the process of shell formation in actively growing pearl oysters that have reached a shell size near 20 mm. If this is the case, then 20 mm is the minimum size at which the fluorochrome calcein can be useful for marking the shell bands of young *P. sterna*. In the brown mussel *P. perna*, Kaehler and McQuaid (1999) reported lower absorbed calcein concentrations in juveniles (fluorescent marker absent) than in adults (fluorescent marker observed), a result that supports our finding that the calcein marker is more effective at larger sizes. This differential success rate may also reflect the limited amount of calcein that small oysters can hold in their paleal cavity when the dye is injected, resulting in a negligible incorporation of calcein into the shell.

The process of calcein incorporation into the shell of marine bivalves has scarcely been studied. Experiments on calcein intake mostly focus on the time the fluorescence marker remains visible in the shell (e.g., Kaehler and McQuaid 1999; Riascos et al. 2007). Since our study was done with juveniles and lasted only a short time, we did not observe degradation of the calcein marker. Its assimilation and incorporation resulted in a mark that spread for 3 to 4 days in the shell of the oysters.

In this study, all the shells displayed autofluorescence (natural fluorescence), which indicates the presence of porphyrins, which are some of the most fluorescent compounds in nature (Guilbault 1973). In molluscs, porphyrins are observed as red, pink, or brown-reddish fluorescence under UV or blue light (Comfort 1949). Usually, the presence of these substances is detected at 390 to 425 nm, depending on the specific structure in a species (Britton 1990). Although Kiefert et al. (2004) reported the presence of porphyrins in *P. sterna* at 400 to 405 nm, we only observed them with a blue-light filter in the range of 330 to 385 nm, and not at 450 to 480 nm.

We counted an average of 15.29 ± 0.26 micro growth bands during the 16-day trial. The small difference between the number of bands counted and the length of the study is most likely caused by variations in the timing of collection of the specimens and the natural light/dark cycle that leads to continuous band formation. The presence of daily growth bands in *Cerastoderma edule* (House and Farrow 1968; Whyte 1975) has been attributed to natural daily light/dark cycles (Pannella and MacClintock 1968; Rhoads and Pannella 1970; Farrow 1972, 1975). These bands can form during the day (Whyte 1975), as well as during the night (House and Farrow 1968).

We found evidence of a sub-daily growth pattern in the form of a very consistent arrangement of 12 layers per micro growth increment, which we suggest corresponds to the arrangement of tabular crystals of aragonite (Fig. 7). The analysis of the factors influencing the formation of these sub-layers was, however, beyond the scope of this study. The existence of a sub-daily growth pattern has also been reported in the bivalves *C. edule* (House and Farrow 1968; Whyte 1975) and *Chione cortezi* (Goodwin et al. 2001; Schöne et al. 2002), although its relation with the physiology and periodicity of growth has not yet been established. In some cases, a sub-daily growth rhythm has been associated with the presence of tides (Evans 1972, 1975; Richardson et al. 1979; Lonne and Gray 1988; Mahe et al. 2010). Rodland et al. (2006) suggested that these sub-daily growth patterns are controlled by internal rhythms or biological clocks, and that these mechanisms are in turn influenced by a complex interaction of physical (water temperature changes, daily light/dark cycles, tides, lunar cycle) and genetic cues (Beetjes and Williams 1985; Richardson 1988; Palmer 1995; Rodland et al. 2006).

From the evidence of this study, we conclude that the micro growth bands appearing in the internal structure of the shell in juveniles of the pearl oyster *P. sterna* are a reliable indicator of their age in days. These bands have a diel periodicity and each band is composed of 12 observable aragonite layers. Finally, the results support the conclusion that staining with the fluorochome calcein is a useful technique to easily and reliably validate the age of *P. sterna* individuals larger than 20 mm in height.

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